On antibiotic resistance

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Chapter 6

Tryptic striptease of *Staphylococcus aureus* unveils the cell surface localization of immunodominant epitopes

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Abstract

The opportunistic pathogen *Staphylococcus aureus* has become a major threat for human health and well-being by developing resistance to antibiotics, and by its fast evolution into new lineages that rapidly spread within the healthy human population. This calls for the development of active or passive immunization strategies to prevent or treat acute phase infections. Since no such anti-staphylococcal immunization approaches are available as yet, the present studies were aimed at identifying new leads for their development. For this purpose, we thoroughly profiled the cell surface-exposed staphylococcal proteome by combining two surface shaving approaches. In parallel, non-covalently cell wall-bound proteins were extracted with KSCN and analyzed by gel-free proteomics, and also the exoproteome was analyzed through gel-free proteomics. Lastly, we screened a selection of the identified cell wall-attached proteins for binding of immunoglobulin G from patients that have been challenged with different types of *S. aureus* over extended periods of time due to chronic wound colonization. The combined results of these analyses highlight particular cell surface-exposed *S. aureus* proteins with highly immunogenic epitopes as potentially powerful targets for the development of protective anti-staphylococcal immunization strategies.

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is an opportunistic pathogen that asymptomatically colonizes approximately 30% of the healthy human population (1-6). Upon invasive growth, *S. aureus* can cause a wide variety of diseases ranging from relatively mild skin infections to severe sepsis. A major reason for concern is the high propensity of *S. aureus* to acquire resistance to antibiotics (7, 8). This is critically underscored by the rapid development of resistance to the antibiotic methicillin. When methicillin was first introduced into the clinic, only methicillin-sensitive *S. aureus* (MSSA) was encountered. However, within two years, the first methicillin-resistant *S. aureus* (MRSA) isolates were observed (9). While MRSA was initially only a threat for hospitalized, elderly and frail individuals, the last two decades have witnessed the emergence of so-called community-acquired MRSA lineages that rapidly spread within the young and healthy population (10-12). Importantly, community-acquired MRSA lineages have now also entered nosocomial settings, which gives rise to increased morbidity and mortality rates. It is therefore a major societal challenge to develop novel, effective and long-lasting anti-staphylococcal therapies (13-16).

In principle, vaccination is a very effective approach for protecting individuals at risk against pathogenic microbes. Unfortunately however, no vaccines against *S. aureus* in general, or MRSA in particular, are currently available (17, 18). While there have indeed been various attempts to develop vaccines for preventing staphylococcal infections, none of the candidate vaccines has successfully passed (pre-)clinical trials. Most likely, this relates to multiple factors, including the ability of *S. aureus* to evade or suppress the human immune system (19-23) as well as the high genome plasticity and adaptability of this wide-spread pathogen (24-29). Also, the published attempts to develop anti-staphylococcal vaccines were so far focused on a relatively narrow group of known *S. aureus* antigens, including capsular polysaccharides and a few cell wall-associated or secreted proteins (17, 18). In this context, it is noteworthy that recent proteomics analyses have indicated the presence of at least 449 different proteins in the *S. aureus* cell envelope (29-34). These proteins may include powerful targets for future anti-staphylococcal immunization strategies, especially if they are exposed to the extracellular environment where they are readily recognizable by the human immune system. However, relatively little is known about the *S. aureus* cell surface-exposed proteome - the 'surfacome' - in terms of the particular protein domains that are directly exposed to the extracellular milieu. Even less is known about the presence of possible immunodominant epitopes within such exposed protein domains that could be used for the development of novel immunization approaches.

To pinpoint candidate targets for novel anti-staphylococcal immunization approaches, the present studies were focused on the identification of immunodominant cell surface-exposed protein domains of *S. aureus*. For this purpose, we first performed an in-depth proteomics

analysis of the surfacome based on the incubation of *S. aureus* cells with immobilized trypsin that cannot penetrate into the cell wall (i.e. 'cell surface shaving' (29)) or with soluble trypsin that can penetrate into the deeper cell wall layers. In parallel, we analyzed proteins released from the cells by spontaneous shedding or by treatment with the chaotropic compound KSCN, and we analyzed the extracellular proteome (i.e. the 'exoproteome') of the investigated cells. Lastly, a screen for immunodominant epitopes in the identified cell surface-exposed proteins was performed using peptide arrays and serum immunoglobulin G (IgG) from patients with the genetic blistering disease epidermolysis bullosa (EB). As shown in previous research, these patients are exposed to multiple and alternating types of *S. aureus* over long periods of time due to chronic wound colonization (35-37). Altogether, the present 'tryptic striptease' of the *S. aureus* cell unveils a set of cell wall-localized and surface-exposed antigens that may serve as targets for novel active or passive immunization approaches to prevent or treat staphylococcal infections.

Materials and methods

Bacterial strains and growth conditions

The *S. aureus* strains Newman, Newman Δ*spa*Δ*sbi* and USA300 were grown overnight in tryptic soy broth (TSB, OXOID) under vigorous shaking at 37C. The cultures were then diluted into pre-warmed RPMI 1640 medium (PAA) to an OD_{600} of 0.05 and cultivation was continued under the same conditions. Exponentially growing cells were again diluted into fresh and pre-warmed RPMI medium to a final OD_{600} of 0.05 and their cultivation was continued to an OD_{600} of 0.2.

Isolation and processing of sub-proteome fractions

Exoproteome **-** Cells were separated from the growth medium by centrifugation (15 min, 6750 × *g*, 4°C). The growth medium fraction thus obtained was filtrated (pore size 0.22 μm), and the exoproteome present in this fraction was precipitated overnight at 4°C with 10% trichloroacetic acid (TCA). Precipitated proteins were pelleted by centrifugation (20 min, 18620 × *g*, 4C) and washed with acetone. Protein pellets were dried and resuspended in 50 mM ammonium bicarbonate and overnight digested with trypsin (Promega) at 37°C.

Surfacome shaving with immobilized trypsin **-** Cells were harvested by centrifugation (10 min, 6080 × *g*, 4°C) and washed twice with PBS containing 40% sucrose (Acros) and 20 mM sodium azide (Sigma-Aldrich). Immobilized trypsin (Pierce) was activated with 50 mM ammonium bicarbonate (Sigma-Aldrich), resuspended in 50 μl PBS with 40% sucrose and 20 mM sodium azide, and added to the washed cells. The shaving reaction was conducted for 45 min at 37°C. Released peptides representing the surfacome were isolated, reduced with 10 mM DTT (30 min), alkylated with iodoacetamide (Sigma-Aldrich), and digested with trypsin overnight at 37°C.

Surfacome shaving with soluble trypsin **-** The protocol for cell shaving with soluble trypsin was essentially the same as the protocol for shaving with immobilized trypsin. In this case immobilized trypsin in 48 µl PBS with 40% sucrose and 20 mM sodium azide was added to the cells.

Spontaneously released proteins **-** The protocol for analysis of proteins spontaneously released from the cells was essentially the same as the protocol for shaving with immobilized trypsin. In this case, 50 µl PBS with 40% sucrose was added to the cells.

Non-covalently cell wall-bound proteins **-** Cells were harvested by centrifugation (10 min, 6750 × *g*. 4°C), washed twice in PBS with 40% sucrose and 20 mM sodium azide, resuspended in 1 M potassium thiocyanate (KSCN), and incubated for 10 min on ice. After centrifugation (10 min, 6750 × *g*. 4°C), the resulting supernatant was filtrated (pore size 0.22 µm) and proteins in the filtrate were precipitated with TCA. The collected proteins were digested with trypsin overnight at 37°C.

Mass spectrometric analyses

Reduction and alkylation, desalting of the samples, mass spectrometric (MS) analysis and database searches were performed as previously described (29). The strain-specific uniprot databases were used for the *S. aureus* strains Newman and USA300, respectively including concatenated reversed databases with 5250 and 5298 entries. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they exceeded the specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.8 and 3.8 for singly, doubly, triply and quadruply charged peptides, respectively. All experiments were conducted in independent triplicates. Peptides were only accepted as identified if they were detected in at least two out of the three replicates per sample set. With these filter parameters no false positive hits were obtained. The identified peptides of strain Newman are listed in Supplementary Table S1 and the identified peptides of strain USA300 are listed in Supplementary Table S2.

Western Blotting

Protein samples were separated using NuPAGE gels (Invitrogen) and separated proteins were subsequently transferred to a Protran nitrocellulose membrane (Whatman) by semi-dry blotting (75 min at 1 mA/cm²). Membranes were incubated with specific antibodies against TrxA and bound antibodies were detected as previously described (38).

Immunofluorescence

S. aureus cells were cultured in RPMI as indicated above, harvested by centrifugation (5 min, 18620 × *g*, 4°C) and washed once in PBS with 20 mM sodium azide. Next, cells were resuspended in 100 µl PBS with 20 mM sodium azide and IsaA-specific (Sakata, Terakubo, & Mukai, 2005) or TrxA-specific antibodies. As a control, cells were incubated with IsaA-specific antibodies that had been pre-incubated with purified IsaA. After 30 min incubation on ice, the cells were collected by centrifugation (5 min, 18620 × *g*, 4°C) and resuspended in PBS with 20 mM sodium azide containing AlexaFluor® 594 labeled goat-anti-rabbit antibodies (Invitrogen). After 30 min incubation on ice, the cells were washing three times in PBS with 20 mM sodium azide, resuspended in MilliQ water and transferred to Polysine-slides (Thermo Scientific). After drying, Vectashield mounting medium (Vector Laboratories) was applied to the slides to prevent photobleaching. Images were recorded with a Leica DM5500 B fluorescence microscope (Leica Microsystems B.V), and image processing was conducted with the ImageJ 1.43m software.

Pepscan analysis

To map regions of cell surface-exposed *S. aureus* proteins that are recognized by human IgGs, we synthesized libraries of linear 15-mer peptides with an overlap of 11 amino acids on solid support (Pepscan), as previously described (39). For some proteins, libraries of CLIPS™ constrained 15-mers were prepared as previously described (40). The peptide libraries were probed with heat-inactivated human sera, in a dilution of 1:1000, with goat-anti-human-HRP conjugate as a secondary antibody, and developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid. A charge-coupled device camera was used to register absorbance at 405 nm. For every single Pepscan dataset, the data was normalized to the average signal intensity of the analysis. Furthermore, the signals for every single protein were normalized to the median of the corresponding protein. In addition the standard deviations of the normalized data sets were calculated for each protein. Peptides with a signal exceeding the median plus twice the standard deviation and a normalized signal intensity higher than three were regarded as being immunogenic domains.

Human plasma

Whole blood samples (12 ml) from EB patients were processed immediately after donation by 1:1 dilution in Hanks' Balanced Salt Solution (Gibco). Plasma was obtained after separation from blood cells using Ficoll-Paque PLUS (GE Healthcare) according to manufacturer's instructions. The collected human plasma was stored at -30°C prior to use. The authors declare that the experiments using human plasma were performed with institutional approval upon the receipt of written informed patient consent, and with adherence to the Helsinki Guidelines.

Results

Complementary protein and peptide identifications in different sub-proteome fractions

To identify protein domains that are exposed on the cell surface of *S. aureus*, a comprehensive gel-free proteomics analysis was performed on the community-acquired MRSA strain USA300 and the MSSA laboratory strain Newman. Specifically, staphylococcal cells were shaved either with trypsin immobilized on agarose beads (Fig. 1A, 'shaving 1') or with soluble trypsin ('shaving 2'). Liberated peptides were collected and subsequently identified through MS. In parallel, gel-free proteomics was applied to define spontaneously released proteins (‘control’), non-covalently cell wall-bound proteins extracted with KSCN ('wall'), and proteins in the exoproteome of the analyzed cells. The rationale of this is approach was that the immobilized trypsin would only access protein domains that stick out from the cell surface, whereas the other approaches would help to distinguish the surface-exposed protein domains from proteins or protein domains that are present in the deeper cell envelope layers or in the exoproteome. Overall, this resulted in the identification of 255 unique proteins from the USA300 strain and 177 from the Newman strain (Fig. 1, B and C). Furthermore, 1191 unique peptides were identified in all samples derived from strain USA300, and 762 in the samples derived from strain Newman (Fig. 1, D and E). Importantly, only two proteins were identified in the control fractions (one protein for each strain), showing that the spontaneous release of proteins from the cells during collection and processing of the different sub-proteome fractions was negligible (Supplementary Tables S1 and S2). Therefore, the control fraction is not further specified in what follows. It is also noteworthy that shaving with immobilized trypsin identified fewer proteins/peptides than shaving with soluble trypsin, suggesting that the soluble trypsin does indeed reach targets in the deeper layers of the cell wall (Fig. 1). The complementarity of the four sub-proteome fractionation approaches is underscored by the relatively small numbers of proteins/peptides that were identified in all samples from each strain (Fig. 1). In fact, this underscores the need to combine multiple approaches for a comprehensive description of the cell wall-, cell surface- and extracellular proteomes of *S. aureus*.

Comparison of KSCN-extracted wall proteins with proteins identified by tryptic shaving

The analysis of the proteins that were extracted from the bacterial wall using KSCN resulted in 369 and 166 different peptides for strains USA300 and Newman, respectively, representing 109 and 49 proteins (Fig. 1, B and D). Interestingly, the number of proteins that are specific for the wall extracts is rather low (19% and 16%) while the number of peptides that are specific for this fraction is much higher (41% and 50%). This indicates that the majority of the cell wall proteins is indeed located at the cell surface, but that only domains of these proteins protrude the whole cell envelope.

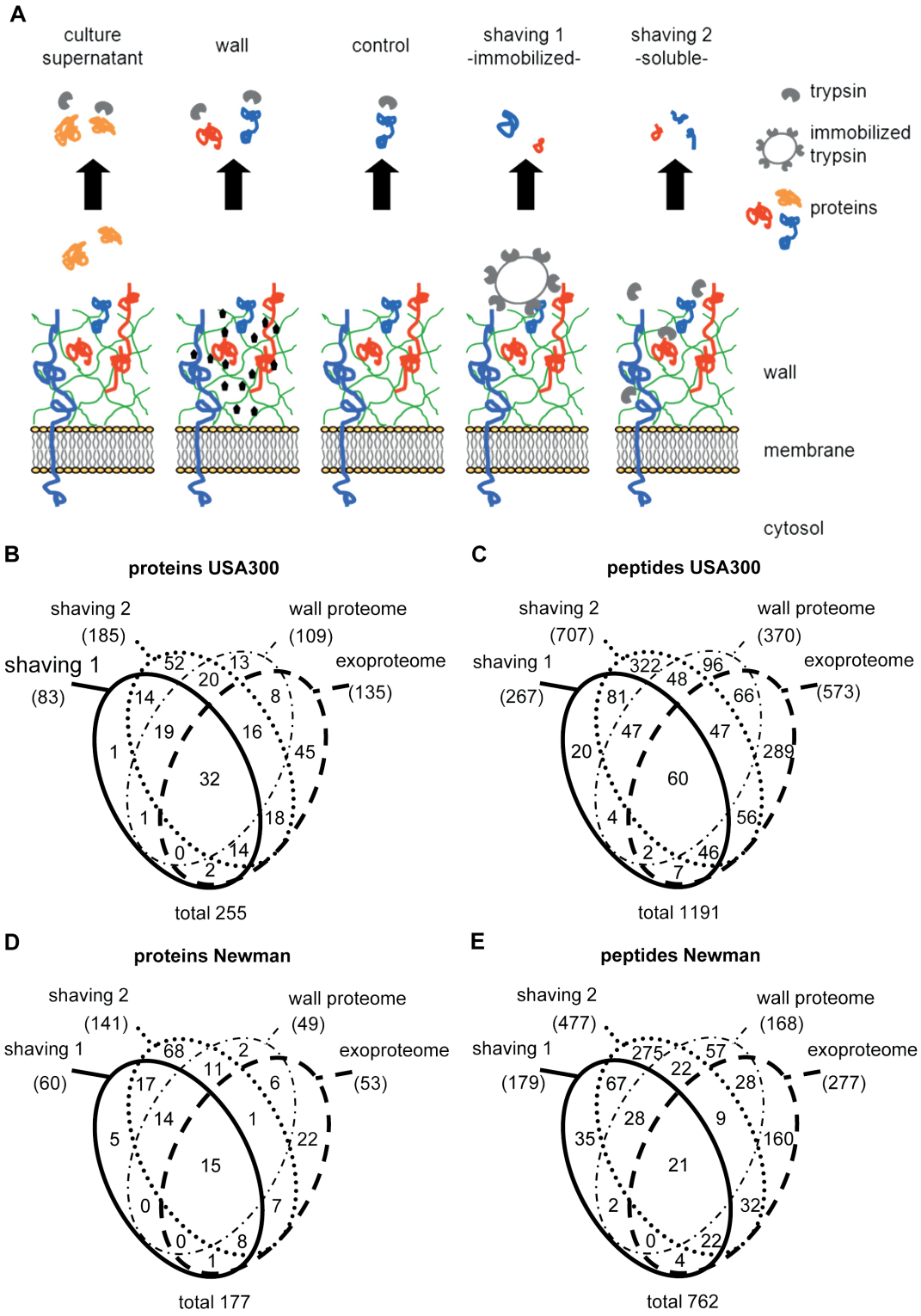

Figure 1. Schematic representation of the analysis of different sub-proteome fractions and proteomics results in numbers. **(A)** Cells were harvested at identical optical densities and proteins in the growth medium fraction ('culture supernatant') were precipitated and digested with trypsin. Non-covalently cell wall-attached proteins were extracted with 1 M KSCN and also digested with trypsin ('wall'). Cells were incubated in PBS with 40% sucrose and 20 mM azide alone ('control'), or in the same buffer with either immobilized trypsin ('shaving 1') or soluble trypsin ('shaving 2'). The Venn diagrams summarize the results obtained for strains USA300 **(B, C)** and Newman **(D, E)** at the protein- **(B, D)** and peptide levels **(D, E)**. 'Shaving 1' and 'shaving 2' respectively mark the results from cell shaving with immobilized or soluble trypsin; 'wall proteome' marks the results from cell wall extraction with KSCN, and 'exoproteome' marks the results from the analysis of culture supernatants.

Comparison of the exoproteome with the wall-attached proteins

In the exoproteome of strain USA300 we identified 573 peptides from 135 different proteins, whereas in the exoproteome of strain Newman we identified only 277 peptides from 53 unique proteins (Fig. 1, B-E). The comparison of these proteins with the proteins identified in the three approaches addressing the wall proteome (wall, shaving 1 and 2) revealed that 30-40% of the proteins are shared between the fractions (50-57% on the peptide level). This comparison reveals that the 'shaving 2' approach has the highest complexity thereby implying either unspecificity of the approach or an extraordinary high complexity of the wall proteome.

Characteristics of proteins in the different sub-proteomes

The *S. aureus* cell wall contains besides the peptidoglycan meshwork also negatively charged teichoic acids. We were therefore wondering whether the possible interaction of positively charged protein domains with negatively charged cell wall components might cause an over-representation of negatively charged protein domains on the cell surface. To address this question, we analysed the p*I* distribution of the identified peptides and proteins in the different subcellular fractions. This analysis revealed bimodal distributions of the p*I*-values of the identified proteins of *S. aureus* strains Newman and USA300, having peaks in the basic as well as the acidic p*I* ranges (Fig. S1, A and B). Interestingly, the p*I* distribution of identified proteins from strain USA300 tended towards the acidic range, while the p*I* distribution of proteins from strain Newman tended towards the basic range. This observation likely reflects the higher number of cytoplasmic protein identifications in all strain USA300-derived samples, as the majority of cytoplasmic proteins exhibit acidic p*I* values. Furthermore, the proteins identified with the shaving approach based on soluble trypsin revealed an under-representation of basic proteins, which can be explained by the higher proportion of cytoplasmic proteins. Nevertheless, the distribution of the *pI* values at the peptide level resembled a unimodal distribution with a peak in the acidic range (Fig. S1, C and D). Especially for the results obtained with samples from strain Newman, we observed a clear over-representation of peptides with a p*I* below 5 and an under-representation of peptides above p*I* 8 for the shaving-based approaches. While ~70% of peptides of the shaving-derived samples have a *pI* below 5, this is only the case for about 40% and 50% for the cell wall-extracted and exoproteome samples, respectively. This indicates that the tryptic shaving approaches preferentially result in the identification of negatively charged peptides. This could either be due to a re-association of positively charged peptides with the negatively charged cell wall during the shaving reaction, or to an over-representation of negatively charged amino acids on the cell surface.

Strain-specific protein identifications in sub-proteome fractions

A comparison of the total protein identifications revealed that the different analyzed sub-proteome fractions from strain USA300 were more complex than those from strain Newman (Fig. 2, Supplemental Figure S2). Furthermore, a search for potential signal peptides, trans-membrane domains and cell wall-binding domains revealed that most proteins that were exclusively identified in samples from strains USA300 or Newman lack such signals, which suggests a predominant cytoplasmic localization (Fig. 2, upper panels). Since about four times more proteins were identified in samples from strain USA300 than in samples from strain Newman, it seems that strain USA300 is more susceptible to lysis. This view is supported by the observation that the bifunctional staphylococcal autolysin Atl - a typical cell wall-bound protein - was identified in the exoproteome of strain USA300, but not in the exproteome of strain Newman. To investigate the presumed lysis of strain USA300, Western blotting experiments were performed in which the localization of the cytoplasmic marker protein TrxA was assessed. As shown in Figure 3A, no TrxA was extracted from the cells with KSCN under conditions that were similar to those applied for cell surface shaving with trypsin. This implies that little if any lysis occurred during the isolation of the different cell-associated sub-proteome fractions. On the other hand, about two-fold more TrxA was detectable in growth medium fractions of strain USA300 than in the equivalent fractions of strain Newman (Fig. 3B). This suggests that strain USA300 is more susceptible to cell lysis than strain Newman, and that cell lysis had occurred already during culturing. This is an important observation, because it implies that the cytoplasmic proteins identified on the surface of staphylcoccal cells used for our present sub-proteome analyses had reached the cell surface during culturing. It should be noticed here that, while we think that autolysis during culturing plays an important role in the apparent export of cytoplasmic proteins to the cell surface and growth medium, we cannot exclude the possibility that some of these proteins are actively exported from the cytoplasm via as yet undefined mechanisms (41).

Proteins common to *S. aureus* strains USA300 and Newman

Despite clear differences, there are also many overlaps in the proteins identified in the respective sub-proteome fractions from *S. aureus* strains Newman and USA300 (Fig. 2, Supplementary Table S3). This is in line with the fact that most of the proteins identified in the present studies are encoded by the genomes of both investigated *S. aureus* strains. Interestingly, seven proteins were identified in all four sub-proteome samples from the USA300 and Newman strains (Supplementary Tables 4 and 5). These are the IgG-binding protein Spa, the secretory antigen SsaA, the transglycosylase IsaA, the fructose-bisphosphate aldolase class 1, the DNA binding protein HU and the ribosomal proteins L29 and L30. To verify cell surface exposure, an immunofluorescence control experiment was performed for IsaA, against which a highly specific

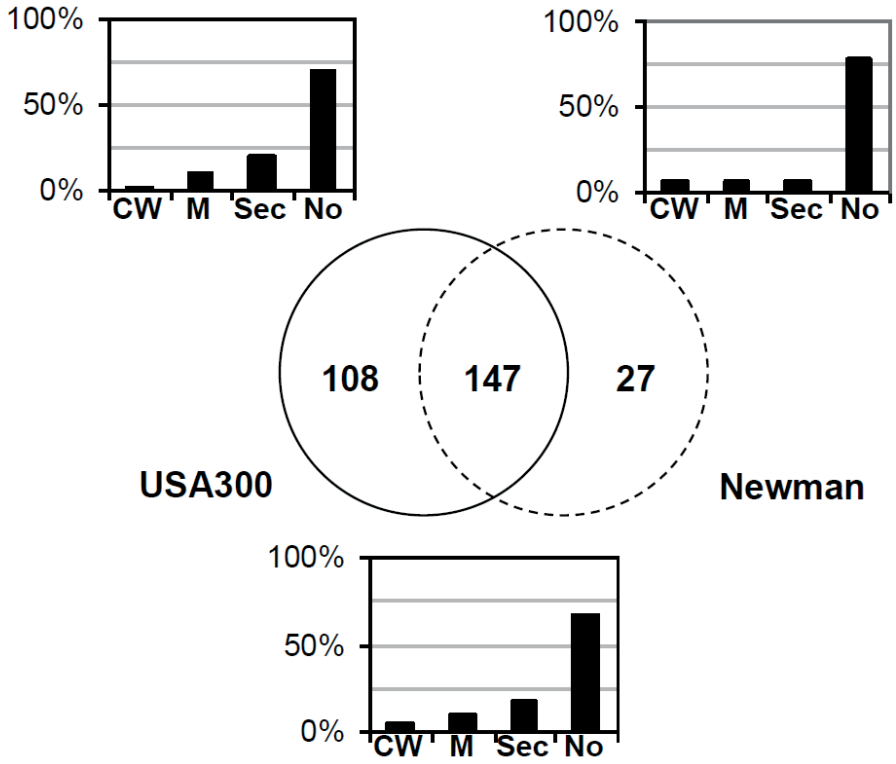

Figure 2. Predicted subcellular localization of the proteins identified for strains USA300 and Newman. The overlapping as well as unique proteins identified in the four sub-proteome fractions from strains USA300 and Newman were analyzed with respect to their predicted subcellular localization as previously described (9). CW, covalently wall-bound proteins; M, transmembrane- and lipoproteins; Sec, secreted proteins; No, proteins with no predicted motif for subcellular localization.

antibody was available. As shown in Figure 3C, the IsaA antibody bound effectively to the cells of a Δ*spa*Δ*sbi* mutant of strain Newman, which lacks the immunoglobulin-binding proteins Spa and Sbi. Titration of the antibodies with increasing amounts of purified recombinant IsaA confirmed the specificity of the IsaA antibody binding to the cells (Fig. 3, D-J). Furthermore, antibodies against the secreted thermonuclease, which was not detected on the cell surface of strains Newman or USA300 did not bind to the cells (Fig. 3K), and the same was true for antibodies against the cytoplasmic marker protein TrxA (Fig. 3l), which was also not identified as being bound to the cell surface (Fig. 3A). Based on these findings, we conclude that the surface shaving approach does indeed yield specific information on the surface exposure of particular *S. aureus* proteins.

Epitope mapping in surface proteins of *S. aureus*

Altogether, our proteomics analyses led to the identification of 281 unique proteins (Supplementary Table 6S, sheet A). Thereof, we selected 54 proteins for further analysis by Pepscan epitope-mapping. The selected proteins include predicted cytosolic, membrane, lipid-modified, cell wall-associated as well as extracellular proteins (Supplementary Table 6S, sheet B). For all of these

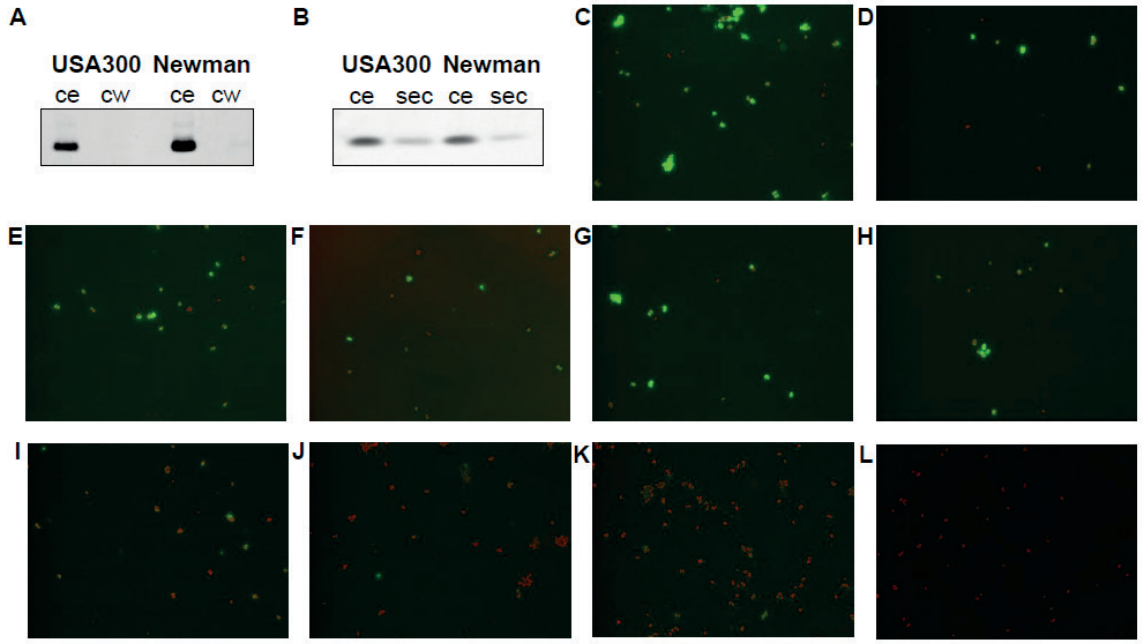

Figure 3. Lysis controls and verification of cell surface localization of IsaA. **(A)** Cells were harvested from cultures with an OD_{600} of 0.2, washed, and non-covalently cell wall-bound proteins were extracted with KSCN. The extracted wall proteins (cw) as well as crude cell extracts (ce) were analyzed by Western blotting with specific antibodies against the cytosolic marker protein TrxA. **(B)** Cultures were harvested at an OD_{600} of 0.2. Cells were separated from the growth medium by centrifugation, and the presence of TrxA in crude cell extracts (ce) or growth medium fractions (sec) was assessed by Western blotting with specific antibodies. **(C)** Cells of *S. aureus* Newman Δ*spa*Δ*sbi* were harvested at OD_{600} 0.2, incubated with an IsaA-specific antibody plus a secondary antibody labeled with AlexaFluor® 594, and inspected by fluorescence microscopy. As a control for specific antibody binding, the IsaA-specific antibody was pre-incubated with increasing amounts of purified IsaA prior to fluorescence microscopy: **(D)** 10 pg IsaA, **(E)** 100 pg IsaA, **(F)** 1 ng IsaA, **(G)** 10 ng IsaA, **(H)** 100 ng IsaA, **(I)** 1 µg IsaA, **(J)** 10µg IsaA. Antibodies directed against **(K)** the secreted thermonuclease Nuc, or **(L)** the cytosolic marker protein TrxA were applied as negative controls for immuno-fluorescence.

proteins linear 15-mer peptide arrays with 11-mer overlaps were prepared. Additionally, arrays with CLIPS™ constrained 15-mers were prepared for the IsaA, LytM and Nuc proteins. Plasma donated by seven different EB patients, who have high IgG responses against staphylococcal proteins (36), was then used for the detection of immunogenic domains. In total, we analyzed the interaction of 6821 peptides with IgG's in the plasma of EB patients. This revealed 358 human IgG-binding peptides from 48 different *S. aureus* proteins. Merging of overlapping sequences finally resulted in the delineation of 201 immunodominant domains (Supplementary Table 6S, sheet C). The most conserved peptides recognized by IgGs from human serum belong to a Zinc-binding lipoprotein (A6QJP6), the extracellular matrix-binding protein (Emp), and the iron-regulated surface determinant (IsdB) (Supplementary Table S6, sheet D; Supplementary Fig. S3). The relative localization of immunodominant protein regions and peptides identified in the different investigated sub-proteomes is schematically presented in Supplementary Figure S4 and Table S7. Notably, the peptides from certain other cell surface-exposed proteins did not bind IgGs from EB patient plasma (i.e. FtsL, RS7, the DNA binding protein HU, a putative thioredoxin, two CsbD-like proteins and an uncharacterized), and these proteins can thus be regarded as negative controls for our epitope mapping analysis.

The highest number of IgG-binding peptides was identified for IsdB, and a close analysis of their location within this protein revealed a highly immunodominant domain between amino acids 47 and 129. This domain was also identified by shaving with immobilized trypsin as being cell surface-exposed (Fig. 4A, 'shaving 1'). A second immunogenic region was detected in the C-terminal part of IsdB between amino acids 395 and 565, which is also identified by surface shaving. Notably, surface shaving with immobilized trypsin ('shaving 1') identified only peptides from the N- and C-terminal regions in IsdB, whereas shaving with soluble trypsin ('shaving 2') also identified peptides from the central region of IsdB. Peptides from the central region were also identified in extracellular IsdB. Thus, the central region of IsdB is not a substrate for immobilized trypsin, suggesting that it is protected by the cell wall.

A remarkable finding was that especially the surface-exposed N-terminal pro-region of the autolysin Atl was very well recognized by the IgGs from EB patients (Fig. 4B). It should be noted that this region is removed from Atl during the processing of the exported pro-Atl into the active amidase and glucosaminidase domains (42). Furthermore, we detected strong immunogenic signals in apparently surface-exposed domains of other proteins, such as the extracellular matrix-binding protein (Emp), coagulase (Coa), fibronectin-binding protein A (FnbpA) and clumping factor B (ClfB) (Supplementary Fig. S4). However, we also observed that for some proteins, such as the chemotaxis inhibitory protein (CHIPS), different protein regions were identified by cell surface shaving and epitope mapping. In the case of CHIPS, we observed an IgG-binding domain in the N-terminus, next to the signal peptide, whereas proteomics identified most peptides in the C-terminal half of this protein (Fig. S4). Interestingly, immunogenic domains positioned next to the signal peptides were observed also for other proteins, such as Atl, Emp, the FPRL1 inhibitory protein (FLIPr), the lipoprotein YkyA, a peptide binding protein (Q2FKI7) and the MHC class II analog protein (Omp7; Fig. S4). Lastly, the epitope mapping revealed also numerous IgG-binding domains in typical cytoplasmic proteins that were found to be exposed on the *S. aureus* cell surface. These include the fructose-bisphosphate aldolase Alf1, the enolase Eno, the triosephosphate isomerase Tim, the elongation factors G and Ts, the ribosomal proteins S5, S13 and L25, the phosphoglycerate kinase Pgk and the glyceraldehyde-3-phosphate dehydrogenase GAPDH (Supplementary Fig. S4).

Discussion

The world-wide spread of highly antibiotic-resistant lineages of *S. aureus* calls for the development of novel anti-staphylococcal therapies. Such therapies could very well include active or passive immunization. Unfortunately, attempts to develop anti-staphylococcal immunotherapy have so far remained unsuccessful, which may relate to the selected antigens. We therefore set out to define cell surface-exposed immunodominant epitopes in two important model strains for *S.*

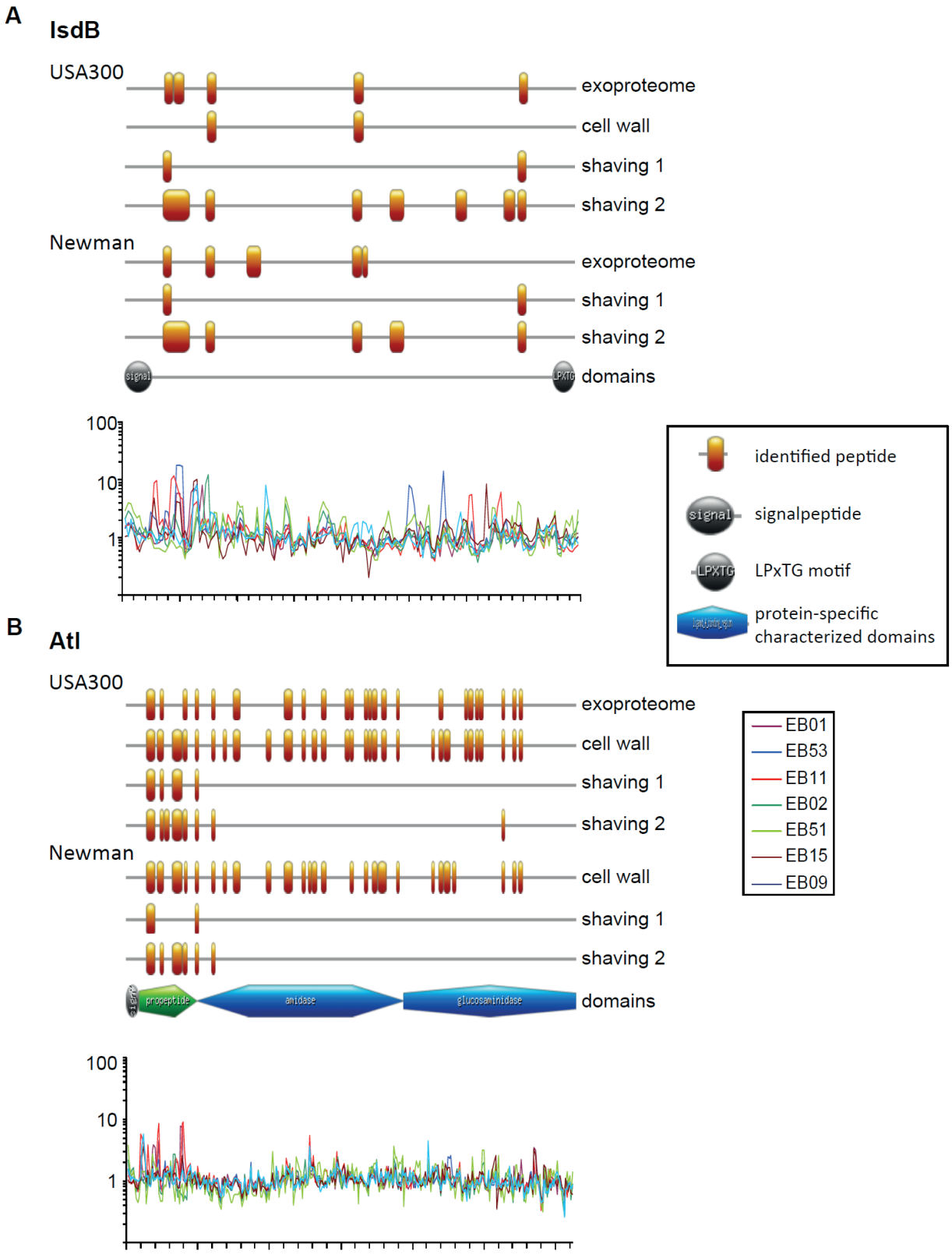

Figure 4. Comparison of proteome and epitope mapping results. Peptides of the IsdB and Atl proteins as identified by proteomics analyses of four different sub-proteomes of *S. aureus* strains USA300 and Newman are highlighted in the linearly depicted protein sequence. In addition known protein domains are indicated. The graphs display the signals from the epitope mapping normalized to the median signal of the respective protein and serum for the seven different EB patients.

aureus, namely the laboratory MSSA strain Newman and the community-acquired MRSA strain USA300.

Various elegant studies on the staphylococcal exoproteome, wall proteome and surfacome were published in recent years (24, 29, 32-34). However, the usefulness of these studies for the rational design of novel antistaphylococcal immunotherapy was limited by the fact that they were based on different *S. aureus* isolates grown in different media under different conditions, and that very different technical approaches were applied (30). Therefore, we designed an integrated workflow in which the cell wall proteomes, surfacomes and exoproteomes of two different strains (Newman and USA300) grown under identical conditions were analyzed in parallel following exactly the same protocol. An important outcome was that, despite substantial differences, there was indeed a significant overlap in the identified proteins of both investigated strains. A major pool of differentially identified proteins was formed by typical cytoplasmic proteins that were detected in and on the wall of both strains. While it is currently debated how such proteins leave the cytoplasm to become localized to the cell wall or the extracellular milieu, it is becoming increasingly clear that some of them have so-called 'moonlighting functions' in virulence (43). In line with this notion, our present studies show that the plasma of EB patients suffering from chronic staphylococcal wound colonization does indeed contain IgGs that specifically bind to a range of cytoplasmic proteins and that there is significant overlap between the IgG-binding domains and domains identified in our proteomic analysis (e.g. Tim, RS5, Eno, GAPDH). Notably, antibodies directed against Eno have been demonstrated to cause opsonophagocytic killing of *S. aureus* and this protein is a known protective antigen on the cell surface of *Streptococcus suis* (31, 44). Furthermore vaccination of rats with recombinant Eno caused protection against dental caries (45). First results also indicate GAPDH as a candidate for a broad spectrum vaccine to reduce infection in aquacultures (46).

The direct comparison of the two different shaving approaches using immobilized and soluble trypsin, respectively demonstrates how different the outcome of the two closely related experiments is. The shaving reaction with the soluble trypsin resulted in the liberation of 2.6 times more peptides representing 2.2/2.35 times more proteins than the assay with the immobilized trypsin. However, the majority of the peptide and protein identifications from the assays with the immobilized trypsin overlap with the results from the experiments using the soluble trypsin. The majority of the protein identifications that were unique for the soluble trypsin shaving approach are cytosolic proteins, like ribosomal and metabolic enzymes and also the pre-protein translocase unit SecA1. This suggests that the soluble trypsin penetrates the whole cell wall and digests also membrane proteins thereby enabling leakage of cytosolic proteins into the environment. However, with the soluble trypsin approach we were also able to identify known cell envelope-associated proteins like penicillin-binding protein 3 (PbpC), the iron-regulated

surface determinant protein IsdB, subunits of the ATP synthetase complex (α, β, b) as well as the iron compound binding protein (Q2FEK8, A6QJC5). In the corresponding negative controls only two peptides were reproducibly identified per strain (Supplementary Tables S1 and S2). From these control experiments we conclude that the shaving assays are essentially free of false-positive identifications due to cell lysis or spontaneous liberation of proteins from the bacterial cell envelope.

Notably, several surface-exposed proteins that were subjected to epitope mapping revealed clusters of epitopes that were specifically localized to certain domains within these proteins. This is best exemplified by the wall-anchored IsdB protein, the autolysin Atl, the adhesin Emp and the transglycosylase IsaA. The significance of these results is underlined by recent efforts to develop an IsdB-based vaccine, which showed that this protein is highly immunogenic (47, 48), and a study in which the binding site of a human monoclonal antibody was located to the IsdB domain between residues 50 to 285 (49). In the present studies, we identified the IsdB domain between residues 47 and 129 as a hot spot for recognition by IgGs from EB patients. This overlap is striking, especially because of the very different approaches that have been applied. Less effort has as yet been put into the development of vaccines containing Emp or Atl, but passive immunization with antibodies against Emp did show a reduction of staphylococcal loads in an animal model (50), and passive immunization with a monoclonal antibody against IsaA gave protection against *S. aureus* in a central venous catheter-related infection model and a sepsis survival model (51). Furthermore, Atl was previously identified as a strong antigen through gel-based immunoproteomic approaches on growth medium fractions of *S. aureus* (52, 53). On this basis, it would be interesting to test the application potential of the N-terminal segment of Atl as a target for active or passive immunization.

Interestingly, the IgGs from different EB patients did not always bind to the same *S. aureus* proteins or protein domains. This may have different reasons. Firstly, the Pepscan approach was based mainly on linear peptides and, therefore, certain conformational epitopes of particular proteins recognized by IgGs of the EB patients might be overlooked. A second reason for the observed variability in IgG responses could be that the different patients did not carry the same *S. aureus* types (36). Thus, it is conceivable that different surface-exposed antigens were either produced at low levels or not at all. This is a realistic possibility since an analysis of 58 different *S. aureus* genome sequences showed large variations in the composition and presence of genes for 25 surface-bound and/or immune-evasive proteins (28). Eighteen of these proteins were identified by our proteomic analyses of strains USA300 and Newman (i.e. ClfA, ClfB, Coa, Eap, Efb, EbpS, Emp, EsxA, FLIPr, FnbpA, IsdA, IsdB, SasG, Sbi, SCIN, SdrD, Spa, VWbp), and 12 were analyzed by our Pepscan approach showing that they were recognized by antibodies from at least one EB patient (i.e. ClfB, Coa, EbpS, Efb, Emp, EsxA, FLIPr, FnbpA, IsdB, SasG, Sbi,

SdrD). It is relevant to note that some of the latter proteins, such as Coa, IsdA, IsdB and Spa, have been implicated as potentially effective targets for immunotherapy (54-56).

Altogether, our present analyses highlight several immunodominant cell surface-exposed proteins of *S. aureus* and specific sub-domains of these proteins as potential targets for novel active or passive immunization approaches. These include the covalently cell wall-bound proteins ClfB and IsdB, a YkyA-like cell wall-binding lipoprotein, the membrane proteins EbpS and LtaS, the non-covalently cell wall-bound and secreted proteins Atl, Sbi, IsaA, Emp, and the cytoplasmic proteins Afl1, Eno, and GAPDH. Future studies will show whether any of these proteins can indeed serve as effective targets for antistaphylococcal immunotherapy.

Author contributions

AD, GB, HG, JWB and JMvD designed the study; AD, ER, MMvdKP, DGAMK, JoB, HPJB, AO, JöB and DB performed the experiments amd analyzed the data; JD, MFJ and MH provided materials and facilities. AD and JMvD wrote the manuscript.

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